THE INFLUENCE OF P-GLYCOPROTEIN ON CEREBRAL AND HEPATIC CONCENTRATIONS OF NORTRIPTYLINE AND ITS METABOLITES

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SUMMARY

The impact of P-glycoprotein (P-gp) on the distribution of nortriptyline (NT) and its metabolites between brain, liver and serum was studied experimentally. The interaction of NT with P-gp *in vitro* was confirmed by measurement of P-gp stimulated ATPase activity (K_m = 257.6 μM, V_{max} = 51.0 nmol phosphate released/mg protein·min). Administration of NT (5 mg/kg, s.c.) to mdr1a knock-out mice resulted in enhanced brain-serum (1.6-fold, p = 0.012) and liver-serum (1.4-fold, p = 0.019) ratios, as compared to the wild-type mice. For a series of NT doses (2.5, 5, 10, 25, 30 mg/kg, i.p.) inhibition of P-gp with cyclosporine A (CsA, 200 mg/kg, i.p.) in rats led to NT brain- and liver-serum ratios that were on average 1.3- (p = 0.005) and 2.1- (p = 0.001) fold higher than those of the controls, respectively. Verapamil (50 mg/kg) (NT, 5 mg/kg) increased the ratios by a factor of 1.6 (p <0.001) and 10.3 (p <0.001) for brain and liver, respectively. Finally, co-administration of methadone (1 mg/kg) did not alter the brain-serum ratio of NT, but in the liver a slight increase (1.5-fold, p = 0.035) was observed. In conclusion, verapamil yielded

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complete inhibition of P-gp at the blood-brain barrier and CsA had an effect corresponding to about 50% inhibition. The results show that P-gp influences the penetration of NT into the brain, and that drug-drug interactions may take place.

KEY WORDS

P-glycoprotein, nortriptyline, methadone, verapamil, cyclosporine A, blood-brain barrier

INTRODUCTION

In recent years it has been shown that the drug-transporting protein P-glycoprotein (P-gp) influences the distribution of drugs across the blood-brain barrier (BBB) /1/. For example, the intra-cerebral concentrations of some opioids are several fold higher in P-gp knock-out mice than in wild-type mice /2/. With regard to many psychotropic drugs, the intra-cerebral concentrations are also higher in P-gp knock-out mice than in wild-type mice, although the difference is generally somewhat smaller than that observed for opioids /3-7/. For tricyclic antidepressants and antipsychotic drugs, relative increases between 30% and 100% have typically been observed in knock-out mice /3-6,8/. For the antipsychotic drug risperidone, however, a 14-fold increase has been reported /3,9,10/.

In this study, we determined V_{max} and K_m for the *in vitro* interaction of the tricyclic antidepressant nortriptyline (NT) with P-gp by applying an ATPase assay. We determined the distribution of NT and three of its metabolites, demethyl-nortriptyline (D-NT), E-10-hydroxy-nortriptyline (E-10-OH-NT), and Z-10-hydroxy-nortriptyline (Z-10-OH-NT), between brain, liver and serum in mdr1a (abcb1a)$^{-/-}$ knock-out mice /11/. We have previously shown that inhibition of P-gp with cyclosporine A (CsA) increased the brain penetration of NT significantly /12/. In the present study, we compared the effect of inhibition with the influence of total absence of P-gp observed in mdr1a$^{-/-}$ knock-out mice. Furthermore, in order to study whether saturation of P-gp might occur at high NT concentrations, we studied the brain-serum ratio of NT at five doses with and without inhibition of P-gp with CsA. By applying a LC-MS/MS assay, it was also

possible to measure the metabolites Z-10-OH-NT and D-NT at most doses. Furthermore, the influence on liver concentrations of NT and the metabolites was examined. In addition to CsA, we applied another strong competitive P-gp inhibitor, verapamil /13/. Studies have indicated that verapamil does interact with P-gp at a different site than CsA /14/, and thus it could be a more potent inhibitor with regard to NT than CsA. Finally, we studied the possible drug-drug interaction effects of the opioid methadone. Methadone is a P-gp substrate /2,7,15/, and as methadone treatment might take place in patients also being treated for depression /16/, we found it of interest to study possible drug-drug interactions between methadone and NT.

MATERIALS AND METHODS

Drugs and chemicals

NT, verapamil, and trimipramine were obtained from Sigma, and the metabolites D-NT, Z-10-OH-NT, and E-10-OH-NT were kindly donated by H. Lundbeck A/S. CsA (Sandimmun) was obtained from Novartis, and methadone was from Nycomed. All other chemicals were of analytical grade.

P-gp-ATPase assay

The transportation of drugs by P-gp is driven by hydrolysis of ATP. Interaction of drugs with P-gp stimulates the associated ATPase activity leading to liberation of phosphate, which can be quantified by a colorimetric method based on the absorption of a 'molybdenum blue' complex, formed upon reduction of an ammonium molybdate-phosphate complex /17,18/.

We assessed the interaction of NT with P-gp using membrane fractions containing human P-gp purchased from Gentest, USA. By adding increasing amounts of NT, the interaction was characterized by the concentration yielding half maximum stimulation (K_m) and the maximum stimulated ATPase activity (V_{max}). Membrane fractions were diluted in TRIS-MES buffer, pH 6.8 (2 mM dithiothreitol, 50 mM KCl, and 5 mM sodium azide) and incubated for 5 min at 37°C with NT. The reaction was started by addition of 4 mM MgATP and stopped by addition of 10% SDS at t = 20 min. A negative control was

stopped at t = 0 min to indicate the amount of phosphate present before the reaction was started. In order to determine the vanadate-sensitive ATPase activity, sodium *ortho*-vanadate was included in the assay. This is an analogue of inorganic phosphate, which inhibits the catalytic cycle of P-gp by forming a stable, non-covalent complex with Mg-ATP at the catalytic site. As a result of this binding only a single turnover of the enzymes occurs. The amount of released phosphate was determined by addition of a colour solution (35 mM ammonium molybdate in 15 mM zinc acetate and 10% ascorbic acid, 1:4 at pH 5.0). The absorbance was read at 750 nm in microtiter plates. A phosphate standard curve and a positive control (20 μM verapamil) were included.

Non-linear regression curves for the relation between concentration and activity were estimated on the basis of the Michaelis-Menten model for enzyme kinetics. K_m and V_{max} values were derived with standard errors (SE) using the computer program GraphPad Prism version 3.0.

Animal experiments

Knock-out mice

Nine male mdr1a$^{-/-}$ (FVB.129P2-*Pabcb1a*^{tm1bor}NT) knock-out mice and nine FVB mdr1a$^{+/+}$ mice (Taconic, Germantown, USA) received NT (5 mg/kg s.c.) dissolved in physiological saline. After one hour they were anaesthetized with halothane, arterial blood was collected, and liver and brain were removed. The blood samples were centrifuged, and serum, liver, and brain were stored at -80°C until analysis.

Interaction with CsA

Fifty-four male Wistar Hannover GALAS rats (Taconic M&B, Ry, Denmark) were divided into five NT dose groups: 2.5 mg/kg, 5.0 mg/kg, 10 mg/kg, 25 mg/kg, and 30 mg/kg. These five groups were further subdivided into two groups that, 2 hours prior to i.p. administration of NT, were treated with either CsA (200 mg/kg, i.p.) (Sandimmun) or a corresponding amount of a control solution (65% water and 35% ethanol). One hour after NT administration the rats were sacrificed as described for the mice.

Interaction with verapamil

Twenty-four rats received verapamil (50 mg/kg) or physiological saline (i.p.) 1 hour prior to NT treatment (5 mg/kg, i.p.). After another hour they were sacrificed as described for the mice.

Interaction with methadone

Twenty-six rats received methadone (1 mg/kg) or physiological saline (s.c.) 1 hour prior to NT treatment (5 mg/kg, i.p.). After another hour they were sacrificed as described for the mice.

Sample preparation

Five hundred mg brain or liver was subjected to mechanical homogenization in 5 volumes of methanol followed by sonication. Fifty µl of internal standard (20 µM trimipramine) was added before homogenization.

Concerning serum samples, 50 µl of internal standard (2.5 µM) was added. In the following, treatment of serum and homogenized tissue was identical. One ml sodium hydroxide (1 M) and 3 ml water were added to 500 µl serum or tissue homogenate. Extraction was performed with 7.5 ml 1.5% isopentylalcohol in n-heptane by shaking for 5 min and centrifuging for 10 min at 3,000 rpm. The solvent layer was transferred to disposable glassware tubes, and 100 µl of 25 mM *ortho*-phosphoric acid was added. The tubes were shaken and centrifuged as described above, the solvent layer was removed, and 10 µl of the water phase was injected into the LC-MS/MS system.

LC-MS/MS procedure

A Quattro Micro instrument (Waters) operating in positive ionization (ESI) mode with a Luna 3µ C8(2) column (Phenomenex) was used for determination of NT, E-10-OH-NT, Z-10-OH-NT, and D-NT. M/Z values were 264.2→233.2 for NT, 280.2→262.2 for E- and Z-10-OH-NT, 250.2→233.3 for D-NT and 295.3→99.7 for trimipramine.

The mobile phase consisted of 0.01 M ammonium formate, pH 3.0. The drugs were eluted using a gradient from 20-70% acetonitrile in 270 seconds, then a plateau at 70% acetonitrile for 30 seconds

followed by another gradient from 70-95% acetonitrile in 60 seconds. The complete run time was 9 minutes. For samples from verapamil treated rats, the mobile phase consisted of acetonitrile and 0.01 M ammonium formate, pH 9.2. For these samples the drugs were eluted using a gradient extending from 40-95% acetonitrile in 390 seconds. The complete run time was 9 minutes. Chromatography was carried out using a flow-rate of 0.3 ml/min with a column temperature of 40°C.

Analysis

Calibration standards were prepared by adding standard solutions to drug-free serum or to methanol prior to homogenization of tissue samples from untreated rats. From 100 to 2,000 nM a quadratic calibration curve based on three standards (100, 1000, and 2,000 nM) was used. Below 100 nM, single point calibration was applied on the basis of the 100 nM level. Tissue concentration was calculated by assuming that the densities of tissue and water are identical.

Absolute recoveries for NT and the metabolites ranged from 45-91%, and the accuracy for NT and metabolites was 96-118% (average measured value as percentage of the spike value). The average coefficient of variation of the controls was 6%.

No endogenous compounds in brain, liver, or serum interfered with detection of any of the compounds, and nor did CsA, verapamil, and methadone or any of their metabolites. Moreover, there was no ion suppression or enhancement.

All results are given as means with SEM (standard error of the mean). Ratios were compared using the non-parametric Mann-Whitney U-test.

This study complies with Danish laws concerning animal experiments and the "Principles of Laboratory Animal Care" (http://www.nap.edu/readingroom/books/labrats/).

RESULTS

***In vitro* interaction with P-gp**

NT stimulated the P-gp ATPase activity, confirming that NT is a substrate for P-gp (Fig. 1). K_m for NT was 257.6 μM (SE = 100), and

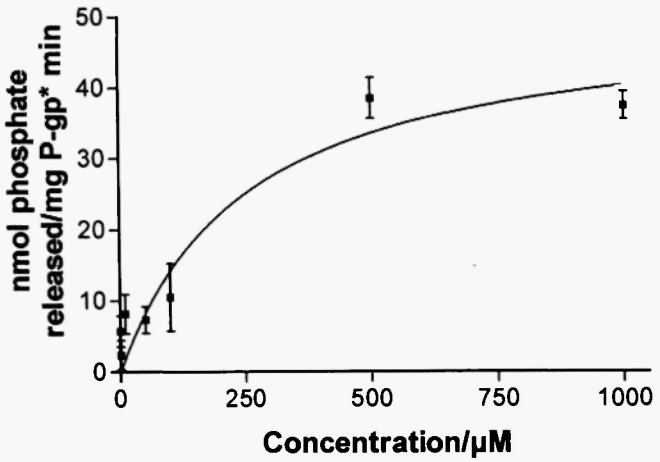


Fig. 1: *In vitro* interaction of nortriptyline (NT) with P-glycoprotein (P-gp). The phosphate release caused by ATP hydrolysis stimulated by P-gp at different concentrations of NT is displayed. Dots represent means of four incubations. The bars show SEM.

V_{max} was 51.0 nmol phosphate released/mg protein·min (SE = 6.7), resulting in a V_{max}/K_m ratio of 0.2 nmol phosphate released/mg protein·min·µM drug.

Brain-serum ratios in knock-out mice

Nine mdr1a$^{-/-}$ knock-out mice and nine control mice were treated with NT (5 mg/kg body weight). The knock-out mice had a mean NT brain-serum ratio of 30 compared to 18 for the control, i.e. a 1.6-fold higher ratio (p = 0.012) (Fig. 2A). The E-10-OH-NT ratio for the knock-out mice was 6.4 times higher than that of the control group (3.05 versus 0.48, p = 0.001). For D-NT, the knock-out mice displayed an average brain-serum ratio of 13 compared to 8.5 for the control group (p = 0.0011). The Z-10-OH-NT concentration in the brain was too low to be measured.

The mean NT liver-serum ratio of the knock-out mice was 1.4 times higher than that of the control group (10.5 versus 7.8, p = 0.019). For the three metabolites no difference between the two groups was observed (Fig. 2B). Serum and tissue concentrations are shown in Table 1.

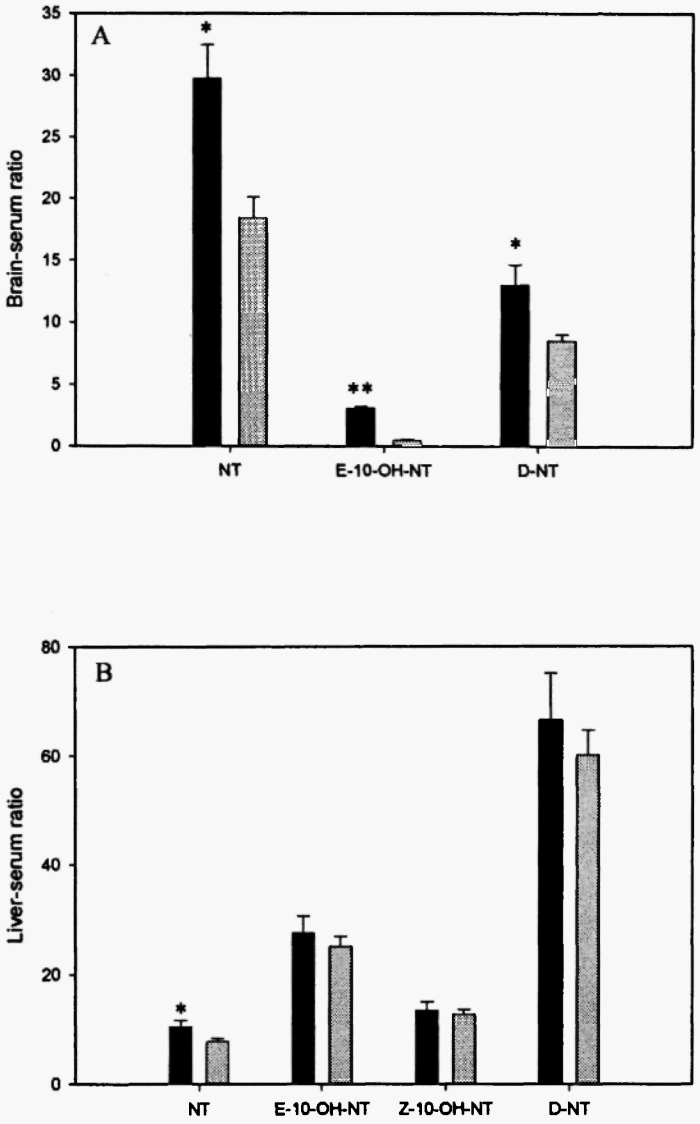


Fig. 2: Tissue-serum concentration ratios in knock-out (black columns) and control mice (grey columns). **A:** Brain-serum ratios. **B:** Liver-serum ratios. The means with SEM as error bars are given. * $p < 0.05$; ** $p < 0.01$. The brain concentration of Z-10-OH-NT was too low to be measured. NT = nortriptyline; D-NT = demethyl-nortriptyline; E-10-OH-NT = E-10-hydroxy-nortriptyline; Z-10-OH-NT = Z-10-hydroxy-nortriptyline.

TABLE 1

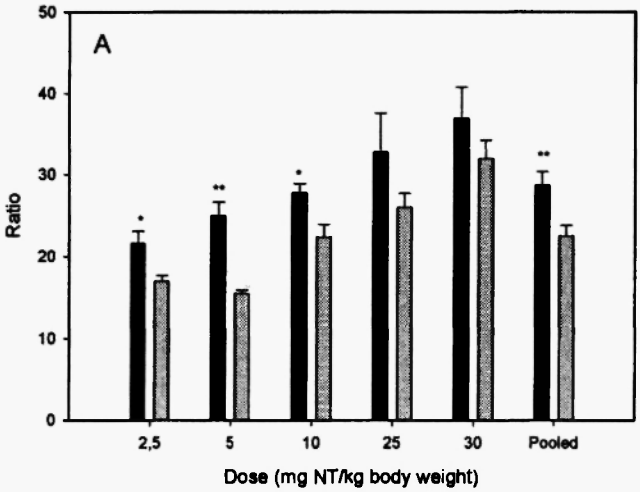
Serum and tissue concentrations in knock-out and control mice

	NT	E-10-OH-NT	Z-10-OH-NT	D-NT
Brain				
Knock-out (n = 9)	21114 (1380)	955 (80)	-	1119 (112)
Control (n = 9)	12074 (599)	116 (22)	-	598 (47)
Liver				
Knock-out (n = 9)	7638 (270)	7674 (321)	3875 (137)	4933 (122)
Control (n = 9)	5117 (210)	6074 (221)	2836 (68)	3954 (147)
Serum				
Knock-out (n = 9)	803 (63)	337 (42)	411 (90)	107 (23)
Control (n = 9)	659 (59)	255 (19)	236 (19)	68 (4)

Results are means (SEM) (nM).
NT = nortriptyline; E-10-OH-NT = E-10-hydroxy-nortriptyline; Z-10-OH-NT = Z-10-hydroxy-nortriptyline. D-NT = demethyl-nortriptyline.

Interaction study with CsA

The rats were treated with five different doses of NT (2.5, 5, 10, 25 and 30 mg/kg bodyweight) and CsA (200 mg/kg) or a control solution. Administration of CsA gave higher brain-serum concentration ratios of NT compared to the control groups (Fig. 3A). For the 2.5, 5 and 10 mg/kg doses, the difference was significant ($p = 0.025$, $p = 0.009$ and $p = 0.047$, respectively), but not at higher doses. A clearly significant difference (1.3-fold, 28.9 versus 22.5; $p = 0.005$) was observed when the ratios were pooled. A tendency towards a relatively smaller difference between the two groups at the higher doses was observed.



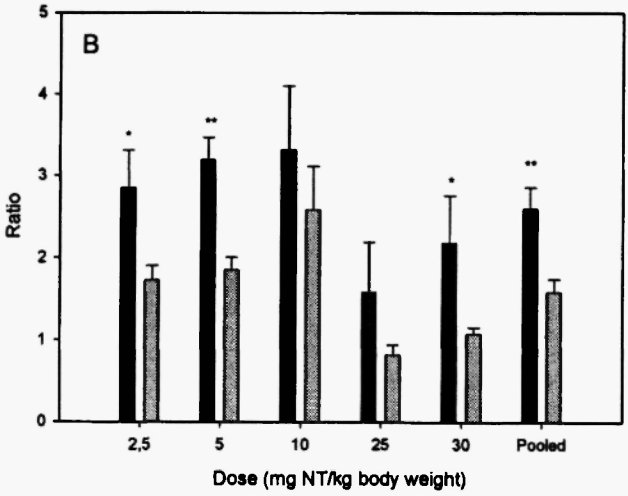


Fig. 3: Mean brain-serum concentration ratios in cyclosporine A treated rats (black columns) and in control groups (grey columns). Error bars denote SEM. **A:** Nortriptyline (NT). **B:** E-10-hydroxy-nortriptyline. The pooled ratios are the average of all dose levels. * $p < 0.05$; ** $p < 0.01$.

The NT liver-serum ratios (Fig. 4A) were highest in the CsA treated groups, and apart from the 25 mg/kg dose (p = 0.065) the differences were significant (p = 0.030 for 2.5 mg/kg, p = 0.047 for 5 mg/kg, p = 0.009 for 10 mg/kg and p = 0.009 for 30 mg/kg). The pooled ratio of the CsA-treated group was 12.5 compared to 5.9 for the control group (p = 0.001).

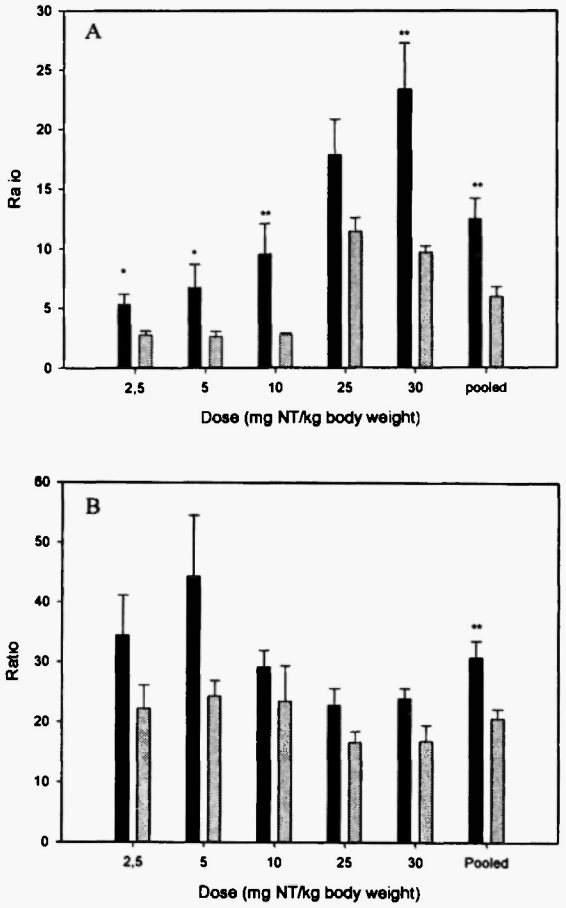


Fig. 4: Liver-serum concentration ratios in cyclosporine **A** treated rats (black columns) and in control groups (grey columns). The means are given with SEM as error bars. **A:** Nortriptyline (NT). **B:** E-10-hydroxy-nortriptyline. The pooled ratios are the average for all dose levels. * p <0.05; ** p <0.01.

TABLE 2

Tissue and serum concentrations (nM) in rats included in the cyclosporine A (CsA) interaction study

	2.5 mg/kg		5 mg/kg		10 mg/kg		25 mg/kg		30 mg/kg	
	+CsA (n=6)	-CsA (n=6)	+CsA (n=5)	-CsA (n=5)	+CsA (n=5)	-CsA (n=5)	+CsA (n=6)	-CsA (n=6)	+CsA (n=5)	-CsA (n=5)
Brain										
NT	4004 (552)	3329 (375)	6024 (465)	6405 (709)	13605 (2394)	12125 (1376)	44635 (5252)	31693 (3129)	56944 (5479)	43188 (6190)
E-10-OH-NT	107 (17)	42 (5)	141 (25)	59 (3)	618 (154)	230 (42)	2415 (903)	978 (49)	4037 (875)	1089 (111)
Z-10-OH-NT	-	-	-	-	47 (9)	26 (6)	230 (15	171 34	402 (119)	77 (14)
D-NT	- -	- -	- -	- -	605 (75)	403 (40)	1771 (179)	1368 (96)	2438 (223)	1521 (186)
Liver										
NT	950 (203)	532 (69)	1623 (407)	1051 (189)	4077 (651)	1522 (208)	25834 (4994)	14281 (2610)	38928 (9707)	13477 (2572)
E-10-OH-NT	1346 (406)	544 (93)	2001 (556)	767 (44)	5551 (1050)	1982 (267)	34686 (4668)	22380 (3590)	49619 (10853)	17228 (3276)
Z-10-OH-NT	-	-	-	-	435 (74)	181 (17)	3490 (514)	1981 (239)	4571 (1144)	1732 (257)
D-NT	- -	- -	- -	- -	1133 (120)	1323 (158)	5634 (873)	3673 (561)	8398 (2010)	5155 (1165)

Serum											
NT	192 (29)	196 (20)	246 (24)	416 (49)	503 (100)	511 (51)	1434 (173)	1257 (214)	1579 (170)	1403 (250)
E-10-OH-NT	42 (7)	26 (5)	46 (11)	32 (3)	202 (46)	98 (19)	1537 (79)	1287 (140)	2067 (418)	1037 (115)
Z-10-OH-NT	- -	- -	- -	- -	18 (4)	13 (3)	137 (10)	125 (19)	157 (22)	83 (10)
D-NT	- -	- -	- -	- -	13 (3)	10 (1)	55 (4)	47 (4)	69 (8)	43 (2)

SEM is shown in brackets.

The serum concentrations of D-NT could not be measured for the 2.5 and 5 mg/kg doses, and likewise the brain and serum concentrations of Z-10-OH-NT were too low to be measured at these two doses

For the main metabolite E-10-OH-NT, treatment with CsA also led to higher ratios compared to the control group for both brain and liver (Figs. 3B and 4B). For the brain-serum ratios the differences were significant for the 2.5 mg/kg dose (p = 0.045), the 5 mg/kg dose (p = 0.009) and the 30 mg/kg dose (p = 0.047). When the ratios were pooled, the difference (2.59 versus 1.59) was clearly significant (p = 0.005). In the liver, the increase in ratios after CsA treatment was not significant for any single dose, but the pooled ratio of the CsA-treated rats (30.8) was significantly higher than that of the control group (20.6) (p = 0.002).

Concerning D-NT and Z-10-OH-NT, it was not possible to determine the serum concentration at the two lowest dose levels, and for the remaining dose levels most differences were non-significant. Likewise the pooled ratios showed non-significant differences.

The actual serum and tissue concentrations are shown in Table 2. It should be noticed that the serum concentrations of NT in the 2.5 mg/kg dose group is at the lower end of the therapeutic interval for humans (190-570 nM) /19/. The 10 mg/kg dose leads to concentrations in the upper part the interval, whereas 5 mg/kg results in concentrations well within the therapeutic interval.

Interaction with verapamil

Rats given NT (5 mg/kg) were treated with verapamil (50 mg/kg) or a control solution. Verapamil treatment gave enhanced NT brain-serum ratios (24.8 versus 15.5, p <0.001) (Fig. 5A). For E-10-OH-NT, verapamil administration somewhat unexpectedly resulted in a significantly smaller brain-serum ratio (0.74 versus 1.63, p <0.001). In the liver, the effect of verapamil was massive, with NT ratios in the verapamil treated rats that were approximately 10 times higher than those observed in the control group (Fig. 5B). The effect on E-10-OH-NT was more moderate, amounting to a 1.8-fold increase (p = 0.005). The serum concentration of NT was within the therapeutic interval in humans /19/ (Table 3).

Interaction with methadone

Twenty-six rats were treated with methadone (1 mg/kg) or a control solution and subsequently with NT (5 mg/kg). Treatment with methadone had no measurable effect on the brain-serum ratios of NT

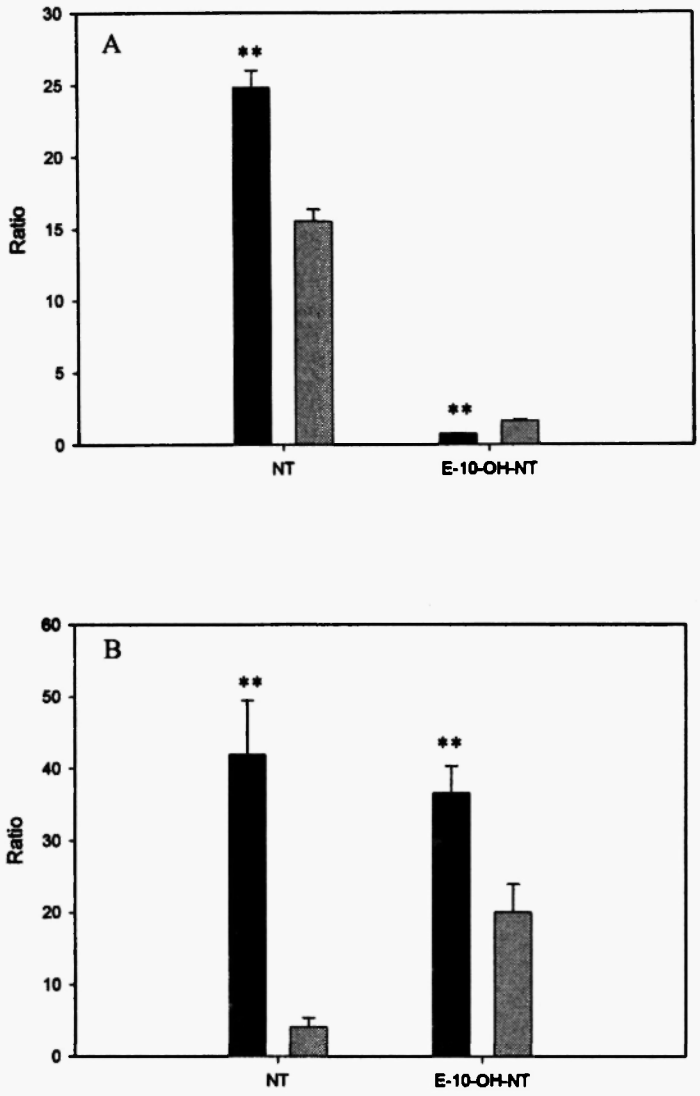


Fig. 5: Interaction study with verapamil. **A:** Brain-serum ratios. **B:** Liver-serum ratios. The black columns refer to the verapamil treated rats and the grey columns to the control group. The means are given with SEM as error bars. * $p < 0.05$; ** $p < 0.01$. NT = nortriptyline; E-10-OH-NT = E-10-hydroxy-nortriptyline.

TABLE 3

Serum and tissue concentrations (nM) in interaction studies with verapamil and methadone

	Brain		Liver		Serum	
	NT	**E-10-OH-NT**	**NT**	**E-10-OH-NT**	**NT**	**E-10-OH-NT**
+Methadone (n = 13)	5132 (683)	76 (10)	1073 (88)	959 (135)	319 (30)	46 (5)
-Methadone (n = 13)	5377 (732)	74 (8)	743 (63)	625 (49)	330 (40)	40 (4)
+Verapamil (n = 12)	9257 (1278)	176 (19)	12854 (1374)	8205 (715)	368 (46)	251 (30)
-Verapamil (n = 12)	4451 (472)	50 (5)	839 (137)	523 (70)	305 (39)	31 (3)

SEM is shown in brackets.
NT = nortriptyline; E-10-OH-NT = E-10-hydroxy-nortriptyline.

and E-10-OH-NT (Fig. 6A). For the liver-serum ratios, however, methadone caused a slight increase for both NT (1.5-fold increase, p = 0.035) and E-10-OH-NT (1.3-fold increase, p = 0.069) (Fig. 6B). The serum NT concentrations were within the therapeutic interval (Table 3).

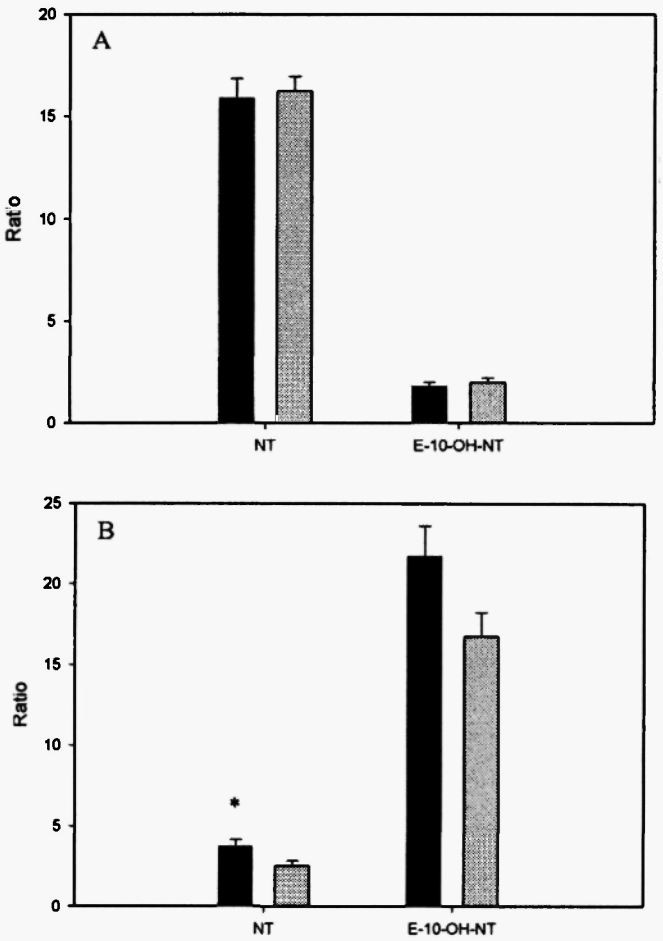


Fig. 6: Interaction study with methadone. **A:** Brain-serum ratios. **B:** Liver-serum ratios. The black columns refer to the methadone treated rats and the grey to the control group. The means are given with SEM as error bars. * p <0.05. NT = nortriptyline; E-10-OH-NT = E-10-hydroxy-nortriptyline.

DISCUSSION

The aspect of drug efflux from the brain mediated by transport proteins has been increasingly appreciated during the last decade /1/. P-gp in particular has been found to be of importance for numerous drugs, including the cardiovascular compounds digoxin and quinidine, HIV protease inhibitors, opioids, and psychotropic drugs /2,4,6,20-22/. In this study we focused on the widely used tricyclic antidepressant NT, which, based on studies in mdr1a and mdr1a/1b knock-out mice as well as inhibition studies in rats, has been shown to be a P-gp substrate /3,4,12/. Furthermore, we focused on the active metabolite E-10-OH-NT, which has previously been considered as an antidepressant in its own right /23/. The present study is an extension of our previous investigation /12/, with inclusion of *in vitro* binding experiments, measurements in knock-out mice, and interaction studies at additional dose levels in order to study the possibility of saturation of P-gp. Moreover, results for the three major metabolites of NT, E- and Z-10-OH-NT and D-NT /24/, were included. Because P-gp can influence the distribution of drugs between liver and blood /8,25/, liver concentrations were also measured.

Using the same *in vitro* approach as Boulton *et al.* /26/, we found that NT had a relatively low affinity for P-gp with a V_{max}/K_m ratio of 0.2. This is of the same order of magnitude as haloperidol (V_{max}/K_m = 0.3) /26/, clozapine (V_{max}/K_m = 0.3) /26/ and 9-hydroxy-risperidone (V_{max}/K_m = 0.4) /10/. The NT ratio is, however, somewhat lower than that of verapamil (V_{max}/K_m = 2.6) and risperidone (V_{max}/K_m = 1.4) /26/.

In knock-out mice the brain-serum ratios of NT were 1.6 times that of the controls, and the liver-serum ratios were 1.4 times higher. The differences are of same order of magnitude as those previously recorded by Uhr *et al.* /4/, who found an NT brain-spleen ratio in mdr1a knock-out mice that was 2.5 times higher that of the controls and a liver-spleen ratio that was 1.4 times higher. Recently, Doran *et al.* /3/ found a mean knock-out brain-serum ratio that was 1.8 times that of the controls. For E-10-OH-NT, we found a profound difference with the knock-out mice having brain-serum ratios that were 6.4 times those of the wild-type mice. This is somewhat higher than the results reported by Uhr *et al.* /4/ (three-fold difference), but still of the same order of magnitude. The main difference between our study and the

study by Uhr *et al.* was the administration of amitriptyline in the latter study instead of NT (NT is the demethylated metabolite of amitriptyline). In the liver, we observed no difference for E- and Z-10-OH-NT between the knock-out and wild-type mice, which is in accord with the results of Uhr *et al.* /4/. Nor did we find any significant effect of P-gp on the liver-serum ratios of D-NT and Z-10-OH-NT.

Mice and rats possess two P-gp genes, called *mdr1a* and *mdr1b* /27/. Only the mdr1a isoform is located at the BBB, thus making the mdr1a knock-out mice a good model for total inhibition of P-gp at the BBB. Both isoforms are, however, present in the liver /28/. It has been reported that hepatic mdr1b expression is upregulated in mdr1a knock-out mice /11/, which might explain the limited effect of absence of P-gp in the liver. Moreover, experiments in knock-out mice generally show that the largest effect on drug distribution is observed in the brain with a smaller effect in the liver /1/.

Our previous study with NT and CsA /12/ suggested that P-gp might be saturated at higher doses. Here we inhibited NT at five dose levels with CsA in order to assess whether there should be a dose dependency of the brain-serum ratio of NT (Fig. 3A). There appears to be a tendency towards a higher ratio in the high-dose area for the control rats, suggesting a saturation tendency. The issue could be further studied by applying higher doses, but since 30 mg/kg gave serum concentrations well within the toxic region, saturation may not be relevant from a clinical point of view.

The pooled brain-serum ratio of NT in the CsA treated rats was 30% higher than in the control group, which is close to the 25% value previously reported /12/. Thus, the relative influence of P-gp inhibition by CsA amounts to about half the effect of a total absence as observed in the knock-out mice. For E-10-OH-NT the 60% increase in brain-serum ratios obtained with CsA treatment also corresponds to our previous result /12/. A CsA dose of 200 mg/kg is rather high, and hence this experiment does not have any direct clinical significance. However, lower doses of CsA have been reported to increase brain-serum ratios of, for example, baicalein (20 mg/kg, 2.5-fold increase), fluphenazine (50 mg/kg, 2.2-fold increase) and nelfinavir (50 mg/kg, 2.7-fold increase), in mice /20,21,29/. Thus, lower doses of CsA can have an impact on drug distribution across the BBB, but considering the low impact of the 200 mg/kg dose on NT in this study, CsA

concentrations in the therapeutic area will probably not give rise to any significant effect on NT brain-serum ratios.

It has been suggested that CsA and verapamil have different binding sites /14/, and hence it was investigated whether a higher degree of inhibition could be achieved with verapamil. Treatment with verapamil yielded a 60% increase in the mean brain-serum ratio of NT, corresponding to the same magnitude as observed in knock-out mice. This indicates that verapamil in the administrated dose is a more effective P-gp inhibitor than CsA with regard to NT. The verapamil concentration in serum was not measured, but a 3 mg/kg dose (i.v.) leads to a plasma concentration (459 ng/ml) /30/ which is higher than the therapeutic concentration in humans (120 ± 20 ng/ml) /31/, and therefore the dose used here is expected to yield plasma concentrations that are higher than the therapeutic level in humans. Thus, the possible clinical relevance of verapamil inhibition of P-gp needs further consideration at lower dose levels. We unexpectedly found that verapamil treatment leads to a significant decrease in the E-10-OH-NT brain-serum ratios. The BBB contains a number of transport proteins, such as organic cation and organic anion transporters, some of which can transport drugs into the brain /32/. Thus, it is possible that one of these actively transports E-10-OH-NT into the brain, and moreover that it can be inhibited by verapamil but not by CsA.

Administration of the P-gp substrate methadone /7/ at a dose expected to give serum concentrations within the range of therapeutic concentrations in humans had no measurable effect on the brain-serum ratios of NT. We have previously shown that therapeutically relevant serum concentrations of risperidone did not increase brain concentrations of NT /12/.

Inhibition of P-gp generally led to increased liver-serum ratios for NT and E-10-OH-NT that were higher than the 1.4- and 1.0-factors observed for knock-out versus wild-type mice. The CsA mediated 2.1-fold increase in NT liver-serum ratios and 1.5-fold for E-10-OH-NT, respectively, corresponds to 150% inhibition of P-gp. Likewise, verapamil led to 10.3- and 1.8-fold increases of the NT and E-10-OH-NT liver-serum ratios, respectively, which are somewhat higher than the ratios of the knock-out mice. As mentioned above, the moderate effect in knock-out mice could be influenced by enhanced expression of the *mdr1b* gene, and since CsA inhibits both isoforms, this can explain why CsA is seemingly able to inhibit P-gp by 150%. Further-

more, there are many transport proteins present in the liver, and the result can be explained by assuming that apart from P-gp another transporter, which can be inhibited by verapamil, can excrete NT into the bile.

Since CsA, verapamil and methadone are metabolised by cytochrome P450 3A4, inhibition of this isoenzyme could also play a role /33/. NT is, however, primarily metabolised by CYP2D6 /34/ and hence major metabolic interactions between NT and CsA and verapamil would not be expected. Methadone is, however, also metabolised by CYP2D6, and metabolic interactions could be of relevance with regard to NT /16/. There seems to be a potential for significant interactions between NT and verapamil in the liver, but studies in which verapamil is administered in more moderate doses are needed to explore this aspect further.

Taking into account the moderate effect observed in knock-out mice and during inhibition with CsA and verapamil, drug-drug interactions at the BBB with regard to P-gp are unlikely to be of major clinical importance for NT. On the other hand, the knock-out mice showed that P-gp influences the brain penetration of E-10-OH-NT to a greater extent than NT. Thus, interactions with E-10-OH-NT could potentially be more important than those concerning NT. E-10-OH-NT is an active metabolite, which in the late 1980s was tried as an antidepressant /35/. The idea was abandoned due to the low brain penetration of E-10-OH-NT, but as the concentration of E-10-OH-NT at steady-state conditions is far larger than in the experiments reported here, further studies might be needed concerning this aspect.

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